AMENDMENTS TO THE SPECIFICATION

1. Insert the following new paragraph on page 4, after line 1:

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

2. Insert the following new section on page 4, after line 19:

Figure 7: We compared the ten-days old-skin organ cultures (C and D) with real human skin (A and B) using H&E (hematoxylin and eosin) stained sections. In general, the organ cultures share very similar morphology both in the epidermis and the dermis connective tissue (compare A to C, and B to D). (E) Confocal microscopy of triple labeled samples shows the differentiation and maturation of epidermis. Keratin is differentially expressed in the basal versus the apical layers much like *in vivo*. WGA (wheat germ agglutinin) labeling shows a thin layer of ECM deposition between epidermis and the connective tissue. (Red: WGA; Green: Keratin; Blue: Nuclei)

Figure 8: (A) Phase-contrast image of fibroblasts (double arrowheads) inside the collagen matrix. Vessel structure is out of focus. (B) Phase-contrast image of the intricate "microvessel" network in the middle of the collagen gel. (mv=microvessel)

Figure 9: (A) Cross section through human breast skin showing a microvessel with a single layer of endothelial cells (arrow) and two peri-endothelial cells (arrowheads). Fibrobroblasts are labeled with double arrowheads. (B and C) Cross sections through a 10-day-old organ culture showing "microvessels" with well defined endothelial cells and with peri-endothelail cells (arrow). Summary: The microvessels in the human skin organ culture are extremely similar to those of the real human skin.

Figure 10: (A) Projection image of a cross section through a 10-day-old organ culture immunolabeled for PECAM (red), a marker for endothelial cells. Cell nuclei are labeled blue. (mv=microvessels) (B) Staining for PECAM in an "in face" area of a microvessel of the organ culture. (C) Projection image of a cross section of the same culture immunolabled for laminin, a major component of the basal lamina, showing that these microvessels are surrounded by a continuous lamina. (D) Immunoblot analysis of cell extracts taken from cultures to show a time-

dependent production/deposition of laminin during microvessel development. Summary: The "microvessels" in the organ cultures express the most characteristic marker for endothelial cells, PECAM, and are surrounded by a continuous basal lamina.

Figure 11: To characterize this organ culture system we used a variety of antibodies to human cell surface proteins and ECM molecules to determine whether this system develops the characteristics of human skin. To detect the presence of glycosylated molecules on the cell surface and on ECM molecules, we used fluorochrome-conjugated lectins that bind specific sugars. Wheat Germ Agglutinin (WGA) (panel A) specifically binds to N-acetylglucosamine and N-acetylneuraminic acid and Concanavalin A (Con A) (panel B) selectively recognizes α-mannopyranosyl and α-glucopyranosayl. Both lectins stain all of the components of the organ culture. We also stained for hyaluronic acid (HA, a glucosaminoglycan that is present in areas of blood vessel remodeling) (panel C) using biotinylated hyaluronic acid binding protein which binds to HA at specific site along the in the molecule. We observed punctated staining in the extracellular matrix regions, in particular in the areas surrounding the microvessels.

Figure 12: Fibroblasts remodel their microenvironment by synthesizing and depositing human ECM molecules to replace the bovine collagen used to initiate the cultures. (A) We detected significant fibronectin (FN) production all over the sandwich culture, but this protein is primarily deposited in the basal membrane of microvessels and its surrounding area. The insert shows a projection view of FN deposition in the microvessel region. (B) Western blot demonstrates that fibroblasts are the major producers of FN. (C) Tenascin was not produced by the cultures except for small pockets of staining in areas close to the blood vessels. (D) Human collagen III is highly produced both in the vessel regions and (E) by the fibroblasts.

Figure 13: Gelatin zymograms showing the activity of remodeling protease activities during the culture maturation. MMP-2 production and activity increase in a time-dependent manner and in the mature cultures it remains at a relatively high level, whereas there is no detectable activity of MMP-9. In the cultures without fibroblasts, the hMVEC were maintained by phobol ester treatment. MMP-2 in these cultures is very low whereas pro-MMP-9 is very abundant. Summary: The "skin" organ culture produces human cell surface and ECM molecules that are glycosylated and produces FN, collagen III and matrix proteases that are known to be involved in remodeling. Tenascin, however, a molecule not found in normal skin is only produced very sporadically in pockets closely associated with the endothelial cells.

Figure 14: When culturing the hMVEC directly to the middle layer of collagen sandwich without dermal fibroblasts, hMVEC can self-organize into the vessel-like structure. But hMVEC will die of apoptosis and the structure will be degenerated after day 3 of plating. Compared to the simple 3D culture, vessel structure in our co-culture system can be maintained for more than 15 days. To explore the survival factors for these vessels, we analyzed the supernatant of different cultures by SDS PAGE silver staining. We detected several lower molecular weight proteins expressed at higher level in our co-culture model (Data not shown). (A) and (B) We verified these proteins as VEGFs and bFGFs by western blot. (C) With confocal imaging, we observed that VEGFs are produced only by endothelial cells of the vessel and the neighboring fibroblasts, and deposited surrounding the vessels. (D) Whereas bFGFs are expressed by fibroblasts in a majority without the correlation of the distance to the vessel.

Figure 15: In our organ cultures, fibroblasts can differentiate into myofibroblasts that associate or are found in very close proximity to the microvessels. α-Smooth Muscle Actin (α-SMA), a differentiation marker protein that is highly expressed in pericytes and the smooth muscle cells that associate with blood vessels, is produced by these peri-endothelial cells. (A) shows an overview of α-SMA localization. Fibroblasts distant from the microvessels do not produce α-SMA. (B) and (C) High magnification confocal images show peri-endothelial cells more clearly. Summary: Under the conditions of our organ culture system fibroblasts in the vicinity of the microvessels differentiate into myofibroblasts.

Figure 16: When the endothelial cells develop into larger vessels we observe the presence of leukocytes inside these vessels (A). (B)-(E) High magnification of regions in (A) to illustrate the presence of these leukocytes. (F) Blood cell immunolabeled with an antibody for macrophage scavenger receptor (mSR), a specific protein for these leukocytes. (G) Blood cells stained with an antibody to CD68 also a cell surface marker for mononuclear leukocytes. Summary: Under our culture conditions, endothelial cells are able to differentiate into mononuclear leukocytes.

Figure 17: IL-8 induces sprouting angiogenesis in the CAM assay. Bright field micrographs of Chorioallantoic Membranes (CAMs). (A) The control membrane shows blood vessels organized in a dendritic pattern. (B) The hIL-8 treated CAM shows that the chemokine stimulates abundant sprouting from the pre-existing vessels (arrowheads). P = Pellet.

Figure 18: Transwell model for IL-8 induced permeability assay. One of the earliest events in sprout formation involves loss of adhesion between the endothelial cells that line the

microvessel. This process is accompanied by an increase in blood vessel permeability. To determine whether hIL-8 stimulates blood vessel permeability we used a culture system that mimics the wall of a blood vessel. Fig. 18 shows a schematic representation of the culture system used to study IL-8-induced permeability. To mimic the angiogenic process *in vivo*, IL-8 was added into the lower chamber to access the endothelial monolayer from the basal side. The permeability was measured as the total amount of FITC light units that leaked into the upper chamber.

Figure 19: Pilot experiment to detect IL-8-induced permeability. In this pilot experiment, we determined the effects of different doses of IL-8 on permeability increase as measured by leakage of fluorecent Dextran into the upper chamber. IL-8 is a potent permeability-inducing agent and its effects are dose-dependent and decrease with increasing concentration of IL-8. The latter effect is characteristic of receptor desensitization and occurs frequently in chemokine receptor function. The phorbol ester PDBu was used as the positive control and DMSO is the vehicle of PDBu.

Figure 20: Dose-dependent effects of hIL-8 on permeability. The results presented in Fig. 19 suggested that concentrations of IL-8 lower than 50ng/ml may be more efficient in stimulating an increase in permeability. Under the conditions of our assay, 50ng/ml IL-8 is the optimal dose and the effects on permeability are comparable to those of 100ng/ml VEGF, the well-known angiogenic factor that causes blood vessel permeability.

Figure 21: Schematic representation of the molecular components of adherens junctions. Maintenance of integrity of the endothelium involves adherens junctions. Critical molecules for maintenance of the integrity of these junctions include the integral membrane proteins vascular endothelial-cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule (PECAM), and their associated cytoplasmic partners, α-, β-, γ- and p120-catenins and SHP2 phosphatase. The adherens junctions are primarily composed of VE-cadherin molecules that bind to each other in the presence of Ca^{++}. Associated with the cadherins are the catenins and actin. In addition, PECAM is also present in these junctions. Loss of adhesion between the endothelial cells can involve phosphorylation of VE-cadherin, PECAM and the catenins.

Figure 22A and B: IL-8 induces VE-cadherin tyrosine phosphorylation. To biochemically characterize IL-8 effects on the hMVEC monolayer, samples treated with 50ng/ml IL-8 were collected at successive times after application of the chemokine and analyzed by immunoblot

for anti-phosphor-tyrosine. We detected strong tyrosine phosphorylation as early as 5 min after treatment. 100ng/ml VEGF served as positive control.

Figure 23: Effects of function-inhibiting antibodies to CXCR1 or CXCR2 on IL-8-induced permeability. Human IL-8 functions through 2 receptors: CXCR1 and CXCR2. Because both receptors are present on endothelial cells, we determined whether either or both are involved the IL-8-induced permeability. To determine which receptor is responsible for the IL-8 effects on permeability, function-blocking antibodies against CXCR1 and CXCR2 were used in the transwell assay. (A) Both antibodies inhibit increase in permeability. (B) Detailed analysis of the earlier stages of permeability increase (first 90 min of treatment) showed that these two antibodies function in different phases. CXCR1 appears to be responsible for the immediate permeability increase and CXCR2 plays a major role in the sustained leakage.

Figure 24: Schematic representation of potential signal transduction pathways stimulated by chemokines upon binding to their seven transmembrane receptors.

Figure 25: Effects of inhibitors of specific signaling molecules on hIL-8-induced permeability. With the transwell model, we explored the downstream signal events that lead to permeability increase. As expected, IL-8 function is inhibited by pertussis toxin, the Gαi inhibitor that blocks the function of most chemokine receptors. We also detected strong inhibition by the PKC inhibitor,Calphostin C, and Src tyrosine inhibitor, Hermimycin. Surprisingly, with the treatment of AG1517, the specific EGFR tyrosine kinase inhibitor, we cannot detect any permeability decrease, even though there is a report indicating that IL-8 downstream signaling depends on the transactivation from GPCR to EGFR.

3. At page 18, line 14, revise the following paragraph to read:

Data and results are shown in Figs. 7-16 ~~Appendix A~~.

4. At page 20, line 29, make the following revisions:

~~Additional data and results are in Appendix B.~~

EXAMPLE 5: ADDITIONAL ANGIOGENESIS STUDIES EMPLOYING THE THREE DIMENSIONAL ARTIFICIAL TISSUE

Chemokines are well known for their functions in inflammation and some CXC chemokines (e.g. IL-8, cCAF) are known to be angiogenic. Our studies on IL-8 using the

Chorioallantoic membrane (CAM) assay show that this chemokine stimulates blood vessel tortuosity and sprouting without leukocyte chemotaxis, strongly suggesting that it directly induces sprouting angiogenesis. We hypothesized that IL-8 functions in the early stages of this process, which involves increase in permeability of the endothelium, basal lamina degradation, and endothelial cell proliferation/migration to form the sprout. To test stimulation of permeability, we used tight monolayers of primary human microvascular endothelial cells on a thin layer of basement membrane-like ECM in transwell units to mimic the wall of a microvessel. Treatment with IL-8 resulted in a dose- and time-dependent increase in permeability that was inhibited by antibodies to IL-8 receptors and by tyrosine kinase inhibitors. Increase in permeability is accompanied by tyrosine phosphorylation of VE-Cadherin and PE-CAM-1, two adhesion molecules that are very important in maintaining the integrity of microvascular endothelium. To investigate IL-8 functions in basal lamina degradation and sprouting we developed a 3D co-culture system that mimics human connective tissue. These cultures develop microvessel-like tubes that have a well-developed, continuous, basal lamina (BL). IL-8 stimulates increase of MMP-9 activity, an enzyme that is important in BL degradation. These findings provide first evidence of the potential molecular mechanisms stimulated by a chemokine in the initiation of the angiogenic process. Because chemokines are major players in pathological conditions that involve inflammation and angiogenesis, our studies provide a basis for development of novel therapeutic targets for angiogenesis, in particular because these small cytokines and their receptors are very amenable to pharmacological manipulations.

Cell cultures: For these studies we used Human primary microvascular endothelial cells (hMVEC; Clonetics), and normal dermal fibroblasts (NHDF; Clonetics). For these studies we used either 24-well plates or transwell units (Becton Dickinson) with polyethylene tetraphthalate filters containing 4 μm pores.

Permeability assays: For these studies we used hMVEC. The filters of the Transwell units were coated, on the upper chamber side, with a very thin coat of matrigel. The thin layer of matrigel allows the cells to adhere well to the filter in an environment that approximates true basal lamina and the very high density forces the cells into a tight monolayer rather than promoting tube formation. The small pore size prevents the cells from migrating through the filters. In this manner we are able to mimic the endothelial lining of blood vessels. Tight monolayers of hMVEC were cultured on the matrigel and treated with different doses of IL-8 from the basal side (lower

chamber). VEGF and PDBu served as positive controls and DMSO as control for the PDBu vehicle. 10 μg/ml of fluorescent Dextran (MW 3000; Molecular Probes) per transwell unit was added at the beginning of the experiment. At each time point, the medium was collected and fluorescence measured with an Hitachi F2000 fluorimeter at Ex 488nm, Em 518nm for 30s. After each collection, an equivalent amount of fresh medium was added.

Connective tissue model: For the molecular studies, hMVEC and NHDF were cultured in 3D and allowed to mature into a tissue that mimics human connective tissue. To prepare these cultures we used either normal 24-well plates or transwell units and sandwiched the endothelial cells between two collagen gels. To make the bottom layer of the tissue, an interstitial collagen matrix of collagens I and III was prepared from Vitrogen (Cohesion Technologies) and the cells evenly suspended in the collagen mixture and applied to the culture well/insert. This layer of collagen was allowed to gel and then was covered with hMVEC. After the hMVEC were attached, the medium was removed and a new collagen gel was prepared the same way as the first gel and applied on top of the hMVEC. Thirty minutes later, after the collagen gelled, 1ml of EGM2-MV medium was applied on top of the sandwich culture in the 24 well plates, or 1ml to the bottom chamber and 0.5 ml to the upper chamber when the transwell units were used. Cultures were maintained at 37°C and of 5% CO2 for at least 2 weeks. Medium was changed 24 hours after the cultures were started and then fed every 48 hours.

Immunoblotting: SDS-PAGE was performed on 10% separating Doucet gels. Protein transfer to nitrocellulose was performed using a wet-transfer apparatus (Bio-Rad) at 100V for 45min. The membranes were blocked for 1h in 5% BSA in TTBS (tweenTBS), incubated overnight at 4°C in primary antibody in 1% BSA in TTBS, washed 3 times for 20min each with TTBS, incubated in anti-mouse or anti-rabbit HRP (1:10,000) in 1% BSA for 1hr, washed as above, and the bands visualized using ECL chemiluminescent kit (Amersham).

Zymography: Zymography was done using 10% denaturing polyacrylamide gels as previously described (Paech and Christianson, 1994). Briefly, gels to test for gelatinolytic activity contained 1% gelatin (Sigma) and were cast 12 hours before the sample separation. Protein samples were separated at 4°C with 10mA constant current for each gel. The gels were renatured in 2.5% Triton-X100 twice (15 minutes each) at room temperature. Renaturation solution was replaced by developing buffer (50mM Tris-HCl, 200mM NaCl, 10mM CaCl2, 0.02% Brij 35, pH7.5), the gels incubated at 37°C for 8-14 hours, fixed (methanol:water:acetic acid, 5:5:1) for 10 minutes, stained

with 0.5% Coomassie Blue for 1 hr and destained (40% methanol plus 10% acetic acid) for an appropriate time to obtain appropriate contrast.

Plasminogen activity assay: A chromogenic plasminogen activator substrate (Boc-Val-Gly-Arg-bNA.AcOH) was purchased from Calbiochem. 50μl of a 10mM stock solution of substrate in water was added to 200μl of 0.1mM HEPES buffer pH 8.0 and heated to 25°C. 25μl cell extract was added to the substrate and absorbance was then read at 405nm.

Results:

Data and results are shown in Figs. 17-25.